Sacace BIOTECHNOLOGIES

HCV Real-TM Quant

Handbook

Real Time Kit for the Quantitative detection of Hepatitis C Virus in human plasma

for use with RotorGene™ 3000/6000/Q (Corbett Research, Qiagen), SmartCycler® (Cepheid), , iQ iCycler™ and iQ5™ (Biorad), MX3000P® and MX3005P® (Stratagene), Applied Biosystems® 7300/7500/StepOne Real Time PCR Systems (Applera), LightCycler 2.0® (Roche), LineGene K® (Bioer), Eco Real Time PCR System® (Illumina)

REF **V1-100/2FRT**

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Σ **100**

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1. NAME

HCV Real-TM Quant

2. INTRODUCTION

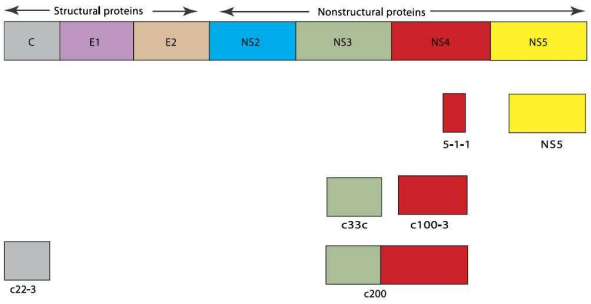
Hepatitis C virus (HCV) is a single-stranded RNA virus that belongs to the family Flaviviridae. In 1974 HCV was initially recognized as non-A, non-B hepatitis virus (NANBH) until 1989 when the etiologic agent was cloned. It is estimated by the World Health Organization that worldwide there are 170 million HCV-infected persons.

The primary mode of HCV transmission is the exposure to infected human blood via intravenous, drug use or unscreened transfusions. Nosocomial HCV transmission during dialysis, colonoscopy, and surgery has also been reported. Perinatal and sexual transmission of the virus is inefficient, but occurs more frequently if the HCV-infected mother or sexual partner is also infected with human immunodeficiency virus type 1.

Most people with acute HCV infection are asymptomatic or have mild symptoms (fatigue, nausea, jaundice) but they are unable to clear the virus and in approximately 80% of cases this leads to chronic infection. In 15 to 20% of patients chronic HCV infection progresses at a variable rate to cirrhosis, with a 1 to 4% annual risk of developing hepatocellular carcinoma.

The discovery of HCV genome in 1989 by Choo et al, paved the way for the development of serological and molecular assays for viral hepatitis C. In the first generation of an enzyme-linked immunosorbent assay (ELISA), wells of microtitre plates were coated with purified recombinant antigen c100-3 which was derived from the non-structural 4 (NS4) region of the HCV genome. However, ELISA-1 was associated with a high percentage (50% to 70%) of false positive results among low-risk blood donors and in the presence of hyperglobulinemia. Thus, second-generation anti-HCV ELISAs were developed. ELISA-2 by Ortho Diagnostics contained recombinant antigens from the core (c22-3), NS3 region (c33c), and NS4 region (c100-3) as well as a part of c100-3, named 5-1-1. Third generation anti-HCV ELISA was introduced in Europe in 1993 and in the USA in 1996. In addition to the antigens of ELISA-2, third-generation anti-HCV ELISA uses a NS5 region antigen of the viral genome. However, synthetic peptide antigens (c22 and c-100) replaced recombinant antigens of ELISA-2. Other manufactures, for example Abbott Diagnostics, used recombinant antigens derived from the same regions of HCV genome. Despite the increased sensitivity and specificity with each generation of ELISA, false-positive antibody results continue to be observed, particularly among low-risk blood donors. Thus, supplemental or confirmatory assays were developed in parallel with ELISA. The recombinant immunoblot assay (RIBA) was used extensively to confirm the presence or the absence of antibody to HCV epitopes. In RIBA, recombinant or peptide HCV antigens are blotted as separate bands onto a nitrocellulose strip flanked by a weak-positive (Level I) and a moderately positive (Level II) strip control.

Fig. 1 Genome organization of HCV and antigens licensed for diagnostic use



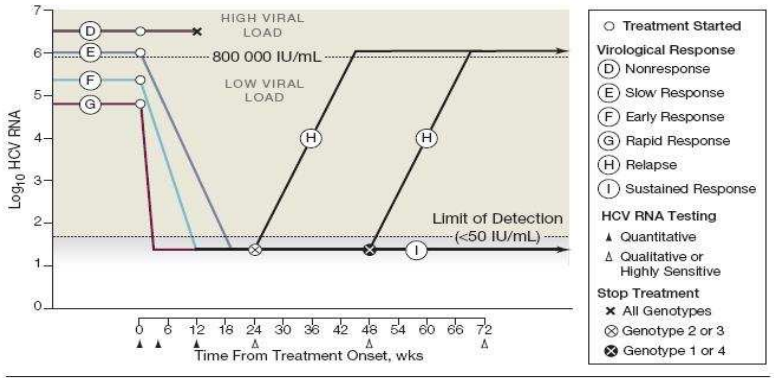
Since ELISA and RIBA are antibody tests, the positivity of either one or both does not necessarily indicate current HCV infection, as patients who have recovered from infection may remain anti-HCV positive for many years. Conversely, during seroconversion, antibody tests may be negative.

The direct molecular biology detection of HCV RNA by reverse-transcriptase polymerase chain reaction (RT-PCR) is considered the gold standard for the diagnosis of HCV infection.

3. INTENDED USE

Kit **HCV Real-TM Quant** is a Real-Time Amplification test for the Quantitative detection of Hepatitis C Virus in human plasma and the simultaneous detection of a HCV-specific Internal Control (IC), by dual color detection. Quantitative HCV RNA testing provides prognostic information regarding likelihood of treatment response and it plays an important role in monitoring the antiviral response to treatment. Sustained virological response is defined as testing negative for HCV RNA, 6 months after cessation of therapy. Recent studies suggest that the rate of response to therapy is also important. For example, conversion to an HCV RNA negative test result after 4 weeks of therapy constitutes a rapid virological response and is a strong predictor of treatment success. Patients who have not had an early virological response, defined as at least a 2-log decline in HCV RNA, after 12 weeks of therapy are unlikely to respond with an additional 36 weeks of therapy and should stop therapy.
The same type of quantitative HCV RNA test should be used throughout a patient's treatment course.

Fig. 2. Monitoring treatment response of patients with chronic hepatitis C Virus Infection



4. PRINCIPLE OF ASSAY

Kit **HCV Real-TM Quant** is a Real-Time test for the Quantitative detection of Hepatitis C Virus in human plasma. HCV RNA is extracted from plasma, amplified using real time amplification and detected using fluorescent reporter dye probes specific for HCV or HCV IC.
Internal Control (IC) serves as an extraction and an amplification control for each individually processed specimen and to identify possible inhibition. IC is detected in a channel other than the HCV RNA. Monitoring the fluorescence intensities during Real Time allows the detection and quantification of the accumulating product without having to re-open the reaction tube after the real time amplification.

5. MATERIALS PROVIDED

Part N° 1 – **"Controls":** RNA isolation controls;
Part N° 2 – **"HCV Real-TM Quant":** Real Time kit;

Contents	
Part N°1 – **"Controls"**[1]	
HCV Rec Pos1 C+ **	4 x 0,01 ml
HCV Rec Pos2 C+ **	4 x 0,01 ml
NCS (Neg. Control Sample) **	4 x 0,5 ml
HCV Rec IC *	4 x 0,16 ml
Part N°2–**"HCV Real-TM Quant"**	
DTT	4 tubes
RT-PCR-mix-1-TM	4 x 0,3 ml
RT-PCR-mix-2-TM	4 x 0,2 ml
Hot Start Taq Polymerase	4 x 0,02 ml
M-MLV Revertase	4 x 0,01 ml
TE-buffer	4 x 0,07 ml
Standard HCV[1]	
• QS1 HCV	4 x 0,025 ml
• QS2 HCV	4 x 0,025 ml
• QS3 HCV	4 x 0,025 ml
Standard IC[1]	
• QS1 IC	4 x 0,025 ml
• QS2 IC	4 x 0,025 ml
• QS3 IC	4 x 0,025 ml

[1] *Standards' and controls' concentrations are specific for every lot.*
**must be used during the sample preparation procedure (see RNA isolation)*
*** must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg; add 90 µl of C– (Negative Control) and 10 µl of HCV Rec Pos controls to the tubes labeled Cpos1 and Cpos2*

6. MATERIALS REQUIRED BUT NOT PROVIDED

- RNA isolation kit (see 11. RNA isolation)
- Desktop microcentrifuge for "eppendorf" type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Tube racks

7. WARNINGS AND PRECAUTIONS

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Use routine laboratory precautions. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas. Do not pipette by mouth.
3. Do not use a kit after its expiration date.
4. Do not mix reagents from different kits.
5. Dispose all specimens and unused reagents in accordance with local regulations.
6. Heparin has been shown to inhibit reaction. The use of heparinized specimens is not recommended.
7. Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
8. Once the reagents have been thawed, vortex and centrifuge briefly the tubes.
9. Prepare quickly the Reaction mix.
10. Specimens may be infectious. Use Universal Precautions when performing the assay.
11. Specimens and controls should be prepared in a laminar flow hood.
12. Handle all materials containing specimens or controls according to Good Laboratory Practices in order to prevent cross-contamination of specimens or controls.
13. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant. Follow by wiping down the surface with 70% ethanol.
14. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
15. Material Safety Data Sheets (MSDS) are available on request.
16. Use of this product should be limited to personnel trained in the techniques of amplification.
17. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification Area. Do not return samples, equipment and reagents in the area where you performed previous step. Personnel should be using proper anti-contamination safeguards when moving between areas.

8. STORAGE INSTRUCTIONS

Part N° 1 – **"Controls"** must be stored at 2-8°C.
Part N° 2 – **"HCV Real-TM Quant"** must be stored at -20°C.
The kit can be shipped at 2-8°C for 3-4 days but should be stored at 2-8°C and -20°C immediately on receipt.

9. STABILITY

HCV Real-TM Quant Test is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

10. SAMPLE COLLECTION, STORAGE AND TRANSPORT

Note: Handle all specimens as if they are potentially infectious agents.

1. EDTA tubes may be used with the **HCV Real-TM Quant**. Follow sample tube manufacturer's instructions.
2. Whole blood collected in EDTA should be separated into plasma and cellular components by centrifugation at 800-1600 x g for 20 min within six hours. The isolated plasma has to be transferred into a sterile polypropylene tube. Plasma may be stored at 2-8°C for an additional 3 days. Alternatively, plasma may be stored at -18°C for up to one month or 1 year when stored at -70°C.
3. Do not freeze whole blood.
4. Specimens anti-coagulated with heparin are unsuitable for this test.
5. Thaw frozen specimens at room temperature before using.
6. Whole blood must be transported at 2-25°C and processed within 6 hours of collection. Plasma may be transported at 2-8°C or frozen.
7. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

11. RNA ISOLATION

The following isolation kits are recommended:

- ⇒ **Ribo Virus 100**– spin column extraction kit (Sacace, REF K-2-C/100)
- ⇒ **Ribo-Sorb-100** (Sacace, REF K-2-1/100

Please carry out the RNA extraction according to the manufacturer's instructions. Add 5 µl of Internal Control during the RNA isolation procedure directly to the sample/lysis mixture (see 12. Internal Control)

12. INTERNAL CONTROL (HCV Rec IC)

HCV Rec IC is a quantitative Internal Control (concentration reported in Data Card) and represents recombinant RNA-containing-structure which carried through all steps of analysis from nucleic acid extraction to PCR amplification-detection. The presence of quantitative HCV Rec IC allows not only to monitor the extraction procedure and to check possible PCR inhibition but also to verify possible losses of the RNA during extraction procedure thus enabling to calculate precisely the HCV viral load.

13. REAGENT PREPARATION

Note: Reaction Mix volume = 25 µl

1. Thaw one set of reagents, vortex and centrifuge briefly the tubes.
2. Prepare reaction tubes or PCR plate.
3. Prepare **Reaction Mix**: add into the tube with **DTT, 300 µl** of **RT-PCR-mix-1**, **200 µl** of **RT-PCR-mix-2, 20 µl** of **Hot Start Taq Polymerase** and **10 µl** of **M-MLV Revertase**. Vortex thoroughly and centrifuge briefly.

*(If it is necessary to test less than 25 samples add into the tube with **DTT, 300 µl** of **RT-PCR-mix-1**, **200 µl** of **RT-PCR-mix-2** and vortex for at least 5-10 seconds. This mix is stable for 1 month at -20°C. Add for each sample (N) in the new sterile tube **12,5*N µl** of mix, **0,5 *N µl** of **TaqF Polymerase** and **0,25*N µl** of **M-MLV.** For the second run it's recommended to import the standard curve generated in the first run, (provided that at least one HCV and IC standards is used)*

4. Add **12,5 µl** of **Reaction Mix** into each tube.
5. Add **12,5 µl** of **extracted RNA** sample to the appropriate tube with Reaction Mix and mix by pipetting.

If the Ribo-Sorb isolation kit is used as a RNA extraction kit, re-centrifuge all the tubes with extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction!

6. Prepare for each run 6 standards and 1 negative control *:
   - add **12,5 µl** of **Quantitation Standards HCV** (QS1 HCV, QS2 HCV, QS3 HCV) into 3 labelled tubes;
   - add **12,5 µl** of **Quantitation Standards IC** (QS1 IC, QS2 IC, QS3 IC) into 3 labelled tubes;
   - add **12,5 µl** of **TE-buffer** to the tube labelled Negative Control;

Close the tubes and transfer them into the Real Time PCR instrument.

14. PROTOCOL DATA ANALYSIS:

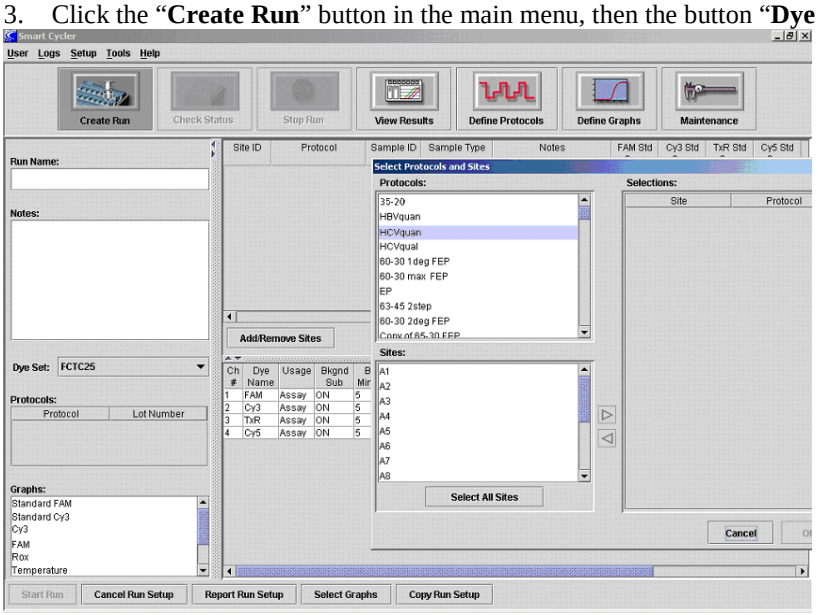
SmartCycler® (Cepheid)

1. Select in the main menu "**Define Protocols**" and press the button "**New Protocol**". Give a name to the protocol and set the following parameters:

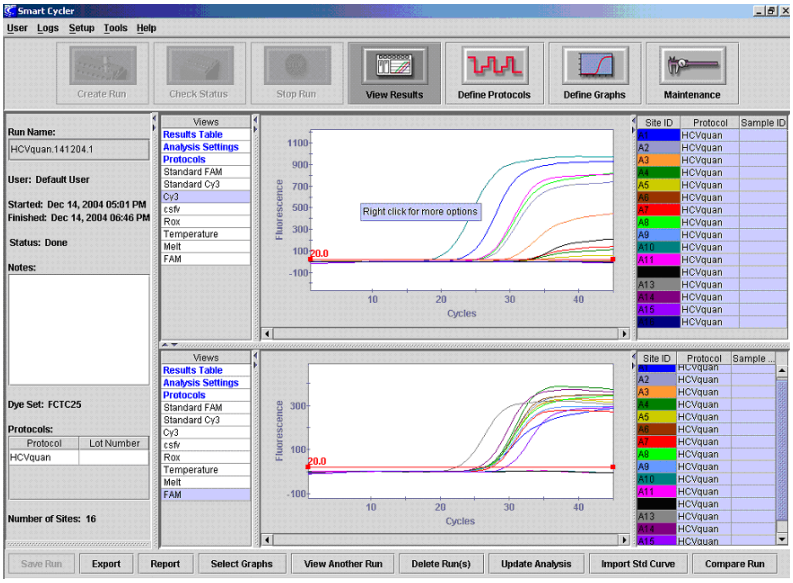
1. Stage1	Hold	50^0C – 1800 sec
2. Stage2	Hold	95^0C – 900 sec
3. Stage3	2-Temperature Cycle	95^0C - 20 sec 60^0C - 40 sec* Repeat – **42** times

*optics ON

2. Choose "**Save Protocol**"
3. Click the "**Create Run**" button in the main menu, then the button "**Dye Set**" and select FCTC25.



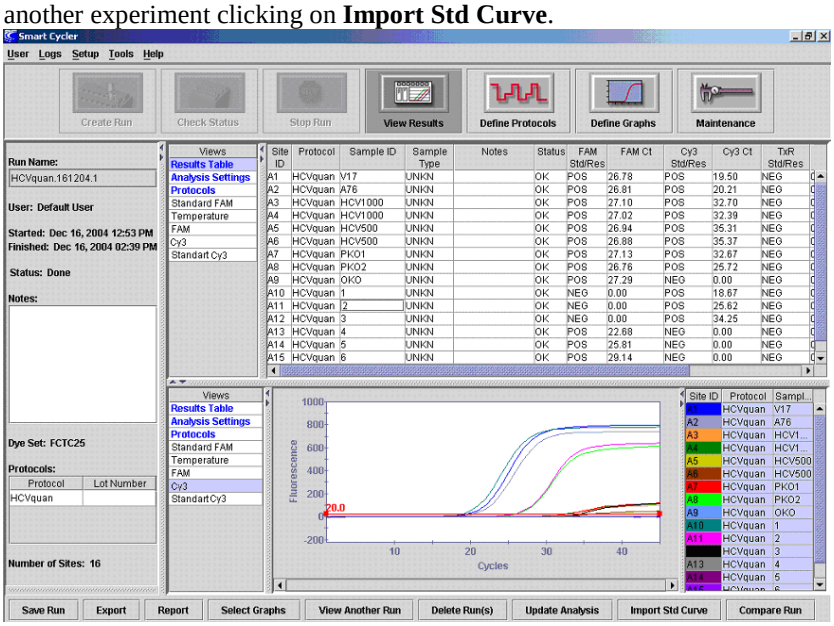
4. Choose "**Add/Remove Sites**" and select in the new windows the protocol and the sites for analysis. Click "**OK**".
5. Choose "**Start Run**" and give a name to the experiment.
6. Insert in the column Sample Type UNKN for samples. Enter the concentrations of the Quantitative Standards (reported on the HCV TM SC Quant Data Card) in the columns Fam Std and Cy3 Std in order to generate Standard curves.
7. Fluorescence is observed in Real Time on the Cy3 channel for HCV RNA and FAM channel for Internal Control.



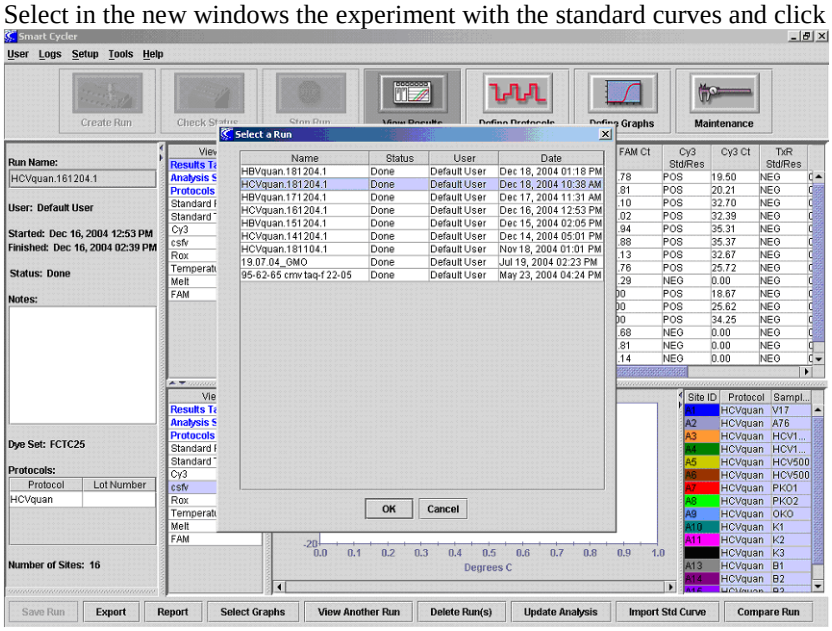
Results Analysis

Choose in the menu "**Analysis settings"** the value **20** for the channels Fam and Cy3. In the table of results (**Results Table**) appear the values of Ct (Threshold cycle) for Fam and Cy3 channels.

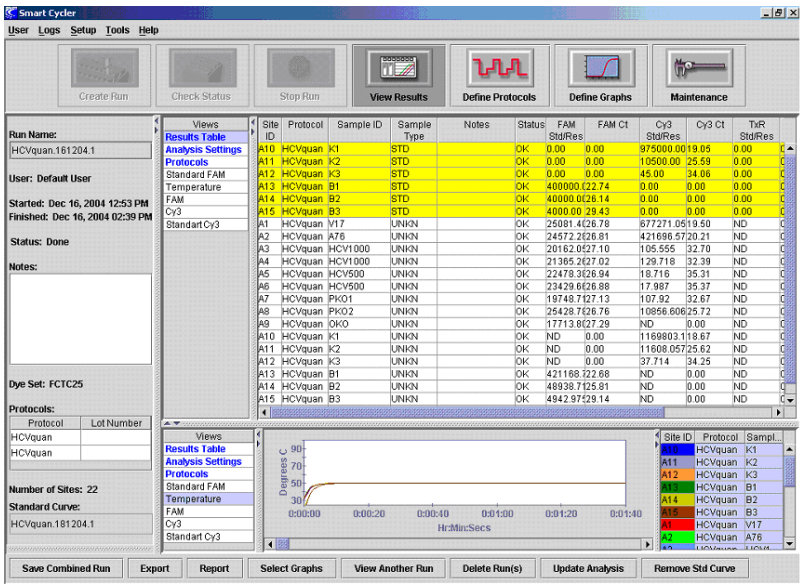
The calculation of HCV RNA concentration in the clinical specimens sample and standards can be performed in the same experiment, but with the SmartCycler software it is possible to calculate the samples concentration by importing the experiment with Standard Curve in the experiment with clinical samples. You can import curves from another experiment clicking on **Import Std Curve**.



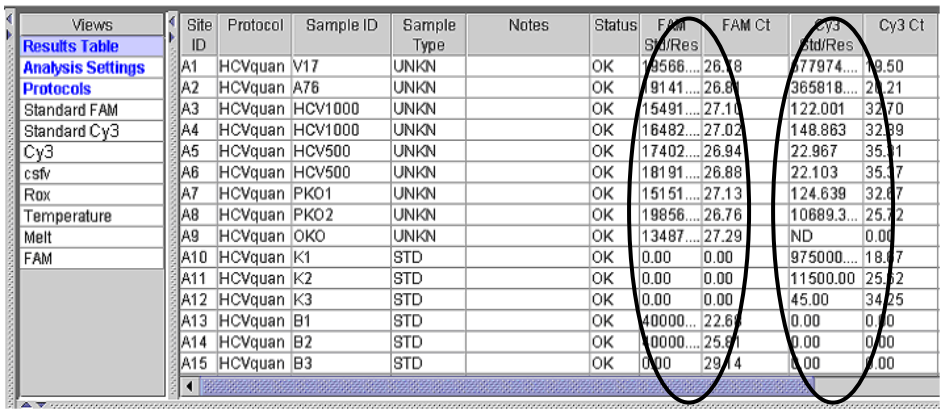
Select in the new windows the experiment with the standard curves and click **OK**



With this technique you can load a standard curve from another experiment.



In any case, if the calibrators were inserted with the clinical samples in the same experiment or after the importation of standard curves from another experiment, in the table of results, in the column FAM Std/Res for IC HCV and in the column Cy3 Std/Res for cDNA HCV should appear the calculated values



Site ID	Protocol	Sample ID	Sample Type	Notes	Status	FAM Std/Res	FAM Ct	Cy3 Std/Res	Cy3 Ct
A1	HCVquan	V17	UNKN		OK	19566....	26.78	677974....	19.50
A2	HCVquan	A76	UNKN		OK	19141....	26.81	365818....	20.21
A3	HCVquan	HCV1000	UNKN		OK	15491....	27.10	122.001	32.70
A4	HCVquan	HCV1000	UNKN		OK	16482....	27.02	148.863	32.39
A5	HCVquan	HCV500	UNKN		OK	17402....	26.94	22.967	35.31
A6	HCVquan	HCV500	UNKN		OK	18191....	26.88	22.103	35.37
A7	HCVquan	PKO1	UNKN		OK	15151....	27.13	124.639	32.67
A8	HCVquan	PKO2	UNKN		OK	19856....	26.76	10689.3....	25.72
A9	HCVquan	OKO	UNKN		OK	13487....	27.29	ND	0.00
A10	HCVquan	K1	STD		OK	0.00	0.00	975000....	18.67
A11	HCVquan	K2	STD		OK	0.00	0.00	11500.00	25.62
A12	HCVquan	K3	STD		OK	0.00	0.00	45.00	34.25
A13	HCVquan	B1	STD		OK	40000....	22.69	0.00	0.00
A14	HCVquan	B2	STD		OK	40000....	25.61	0.00	0.00
A15	HCVquan	B3	STD		OK	0.00	29.14	0.00	0.00

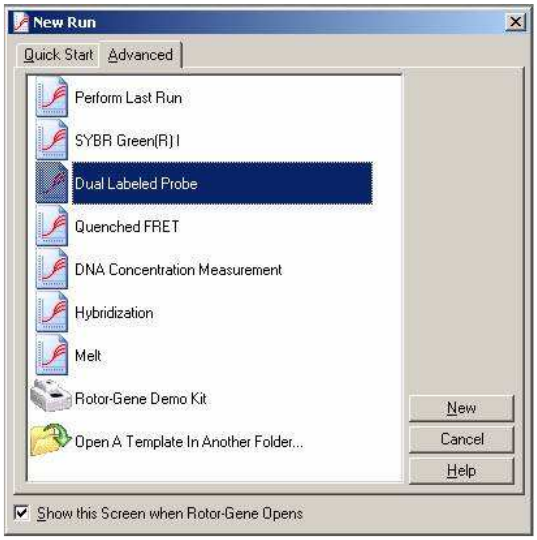
Views: Results Table, Analysis Settings, Protocols, Standard FAM, Standard Cy3, Cy3, csfv, Rox, Temperature, Melt, FAM

Abbreviations:

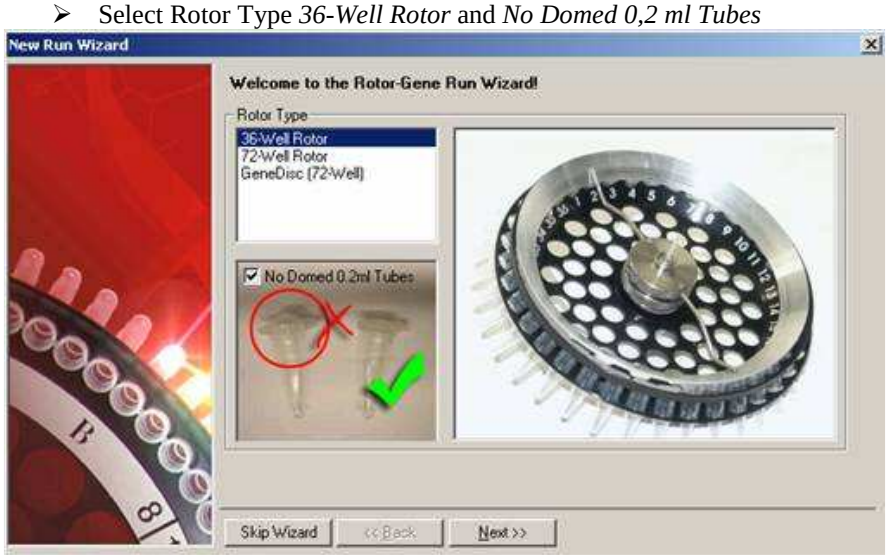
UNKN – Samples, STD – Standards, PKO1, PKO2 – HCV Positive Controls, OKO – Negative Control Sample, K1, K2, K3 – HCV Quantitative Standards, B1, B2, B3 – IC Quantitative Standards

RotorGene™ 3000/6000/Q (Corbett Reasearch, Qiagen)

1. Select *New Run* and *Dual Labeled Probe*. Click *New*.

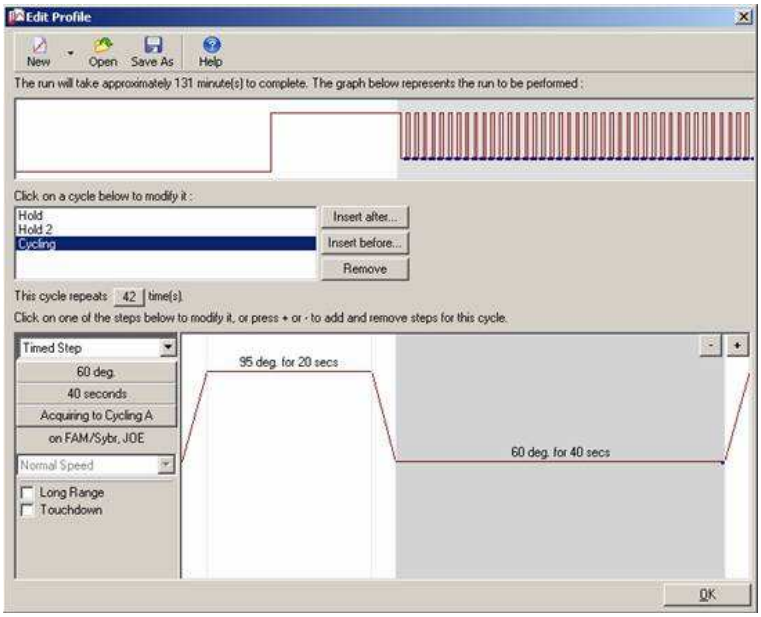


2. Program Rotor-Gene 3000/6000/Q as follows:
   - Select Rotor Type *36-Well Rotor* and *No Domed 0,2 ml Tubes*

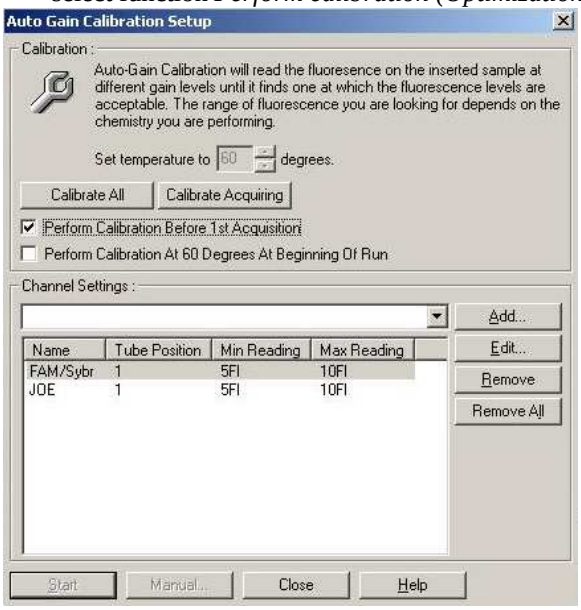


   - *Reaction Volume* (**µl**): **25**
   - *Temperature Profile*:
     Hold: 50°C – 30 min
     Denature: 95°C – 15 min
     Cycling – 95°C – 20 sec
     60°C – 40 sec
     Cycle Repeats – 42 times

Fluorescence is measured at 60°C on FAM (Green) and JOE (Yellow) channels.



3. Press the button *OK*.
4. In the window *New Run Wizard* click *Calibrate* (Gain Optimisation…for Rotor-Gene 6000). In the new window *Channel Setting* select channels *Joe (Yellow)* and *Fam (Green).* Indicate *Min Reading* **5**, *Max Reading* **10** and select function *Perform calibration (Optimization) before 1st Acquisition.* Click the *Close* button.


Auto Gain Calibration Setup
Calibration :
Auto-Gain Calibration will read the fluoresence on the inserted sample at different gain levels until it finds one at which the fluorescence levels are acceptable. The range of fluorescence you are looking for depends on the chemistry you are performing.
Set temperature to 60 degrees.
Calibrate All
Calibrate Acquiring
Perform Calibration Before 1st Acquisition
Perform Calibration At 60 Degrees At Beginning Of Run
Channel Settings :
Add...
Edit...
Remove
Remove All
Name
Tube Position
Min Reading
Max Reading
FAM/Sybr 1 5FI 10FI
JOE 1 5FI 10FI
Start
Manual...
Close
Help

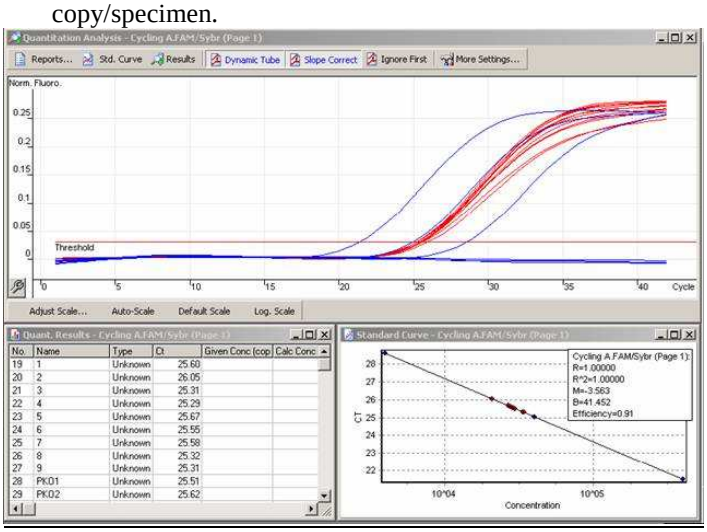
5. Select *Next* and click *Start run*

Program the position of the tubes in the carousel of the Rotor-Gene 3000/6000/Q and enter the concentrations of the Quantitative Standards (reported on the HCV TM RG Quant Data Card) in order to generate Standard curves.

Data Analysis

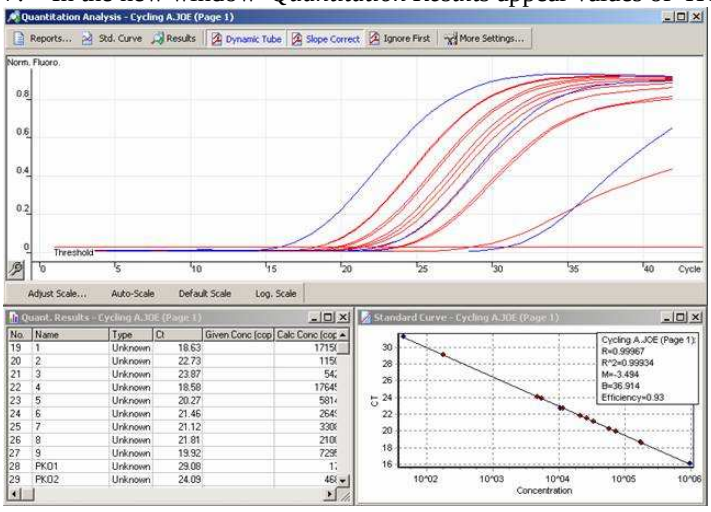
IC amplification analysis (*Cycling A.Fam(Green*)

1. Press *Analysis* and then select *Quantitation* →*Cycling A.Fam* (*Cycling A.Green*)→ *Show*
2. Turn off the automatic option *Threshold.*
3. Press the buttons *Dynamic Tube*, *Slope Correct*
4. In the table of results (*Quantitation Analysis*) select *More settings* and set *NTC Threshold* to 10%
5. Select *Threshold*: 0,03
6. In the table of results (*Quantitation Results*) appear the values of Ct (Threshold cycle) which should be ≤ 30.
7. In the menu window *Quantitation Results*, column *Calculation concentration*, appear values of IC cDNA copy/specimen.



HCV amplification analysis (*Cycling A.Joe (Yellow)*

1. Press *Analysis* and then select *Quantitation* →*Cycling A.Joe* (*Cycling A.Yellow)* → *Show*
2. Turn off the automatic option *Threshold.*
3. Press the buttons *Dynamic Tube*, *Slope Correct*
4. In the table of results (*Quantitation Analysis*) select *More settings* and set *NTC Threshold* to 6%.
5. Select *Threshold*: 0,03
6. In the table of results (*Quantitation Analysis*) appear the values of Ct (Threshold cycle).
7. In the new window *Quantitation Results* appear values of HCV cDNA copy/specimen.



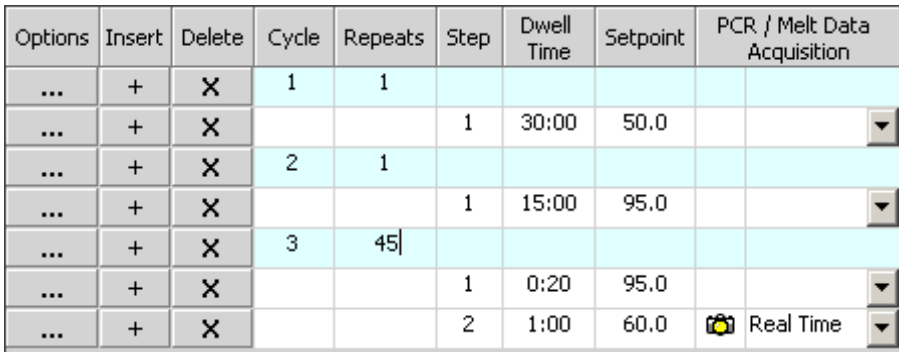
iQ iCycler™ and iQ5™ (Biorad)

Make sure that the iQ5 instrument is calibrated for working with 50 µl reaction mix. Perform the calibration with the same tubes in which the amplification will be carried out.
Make sure that the following settings for **dynamicwf** are selected:

1 cycle 95 °C – 30 s
1 cycle 95 °C – 30 s*
*fluorescence measurement on the second step

1. Select in the main menu "**Define Protocols**" and click "**Create a new protocol**". Set the following parameters:

Cycle	Repeats	Step	Dwell Time	Set Point
1	**1**	**1**	**30:00**	**50.0**
2	**1**	**1**	**15:00**	**95.0**
3	**42**	**1**	**00:20**	**95.0**
		2	**01:00**	**60.0**



2. Select "**Edit Plate**" for iQ5 or **"View/Save Data"** for iQiCycler to create the "plate" for samples and controls. Enter the concentrations of the Quantitative Standards (reported on the HCV TM iQ Quant Data Card) in the "**Standard Quantity**" box (QS1 HCV, QS2 HCV, QS3 HCV as **Standard** in the **HEX** channel and **Unknown** in the **Fam**; QS1 IC, QS2 IC, QS3 IC as **Standard** in the **Fam** channel and **Unknown** in the **HEX** channel). Use icon "Unknown" for all samples.
3. Choose the **FAM** and **HEX** channels for all samples.
4. For the iQ5 instrument enter the reaction volume "**Sample volume**" **25 µl**. Select caps type "**Seal Type**" and tubes type "**Vessel Type**". Save plate setup.
5. Begin the amplification:
   For the iQ iCycler instrument activate the key "**Run with selected plate**" and choose in the window of the opened menu, the reaction volume **25 µl**, select "**PCR Quantification Melt Curve"** and "**Experimental Plate**". Click the button "**Begin Run**" and save the experiment settings.
   For the iQ5 instrument activate the button "**Run**" and choose in the window of the opened menu "**Collect Well Factors from Experimental Plate**". Click the button "**Begin Run**" and save the experiment settings.

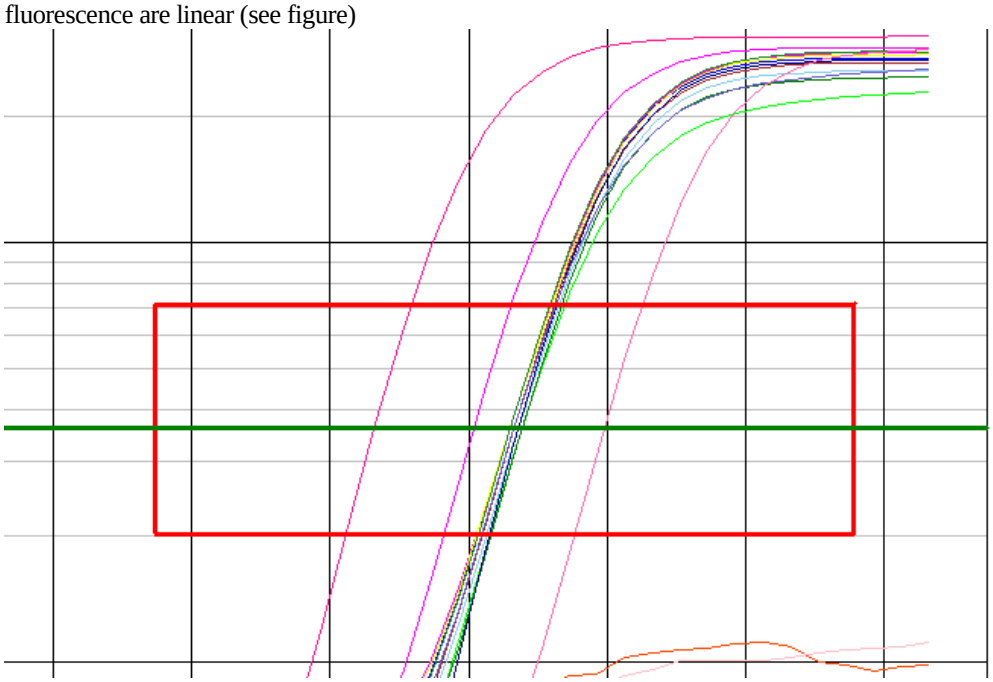
Data Analysis

The results are interpreted with the software of "iQ iCycler" or "iQ5" through the presence of crossing of fluorescence curve with the threshold line. Internal Control (IC) is detected on the FAM channel and HCV RNA on the HEX channel

Activate the button "**PCR Quant**" for the results analysis

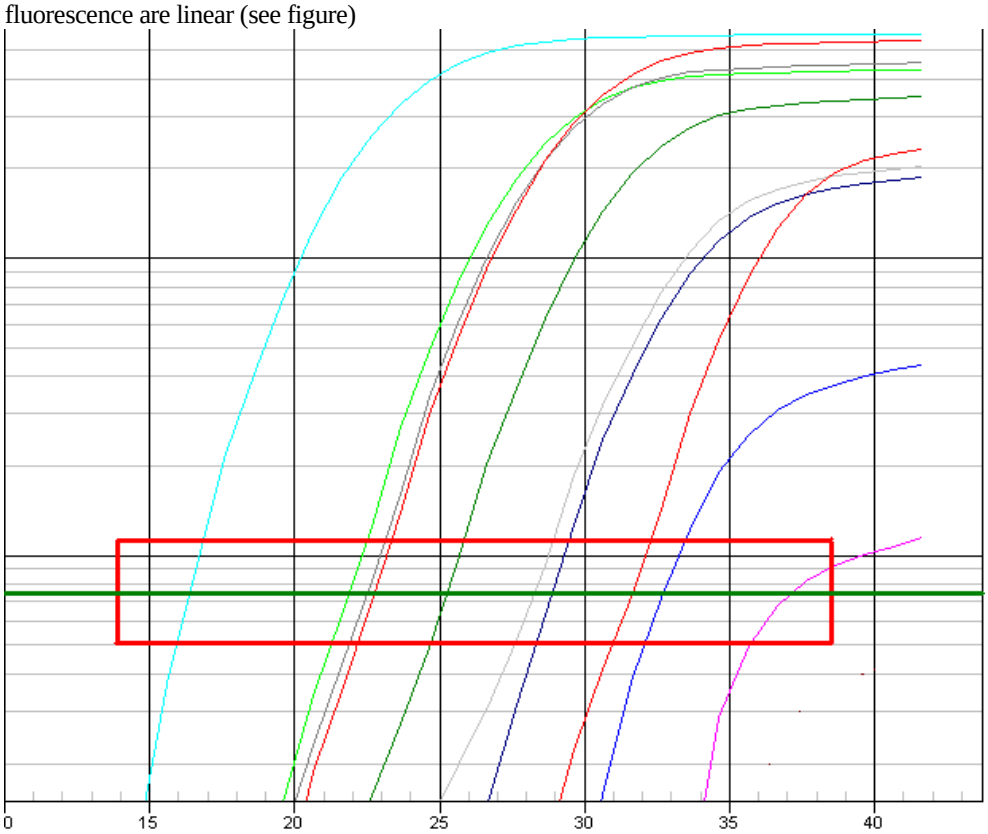
Results analysis for **Fam** channel (IC)

Activate the button "**Log View**". Put the threshold line (with the left button of the mouse) at such level where curves of fluorescence are linear (see figure)



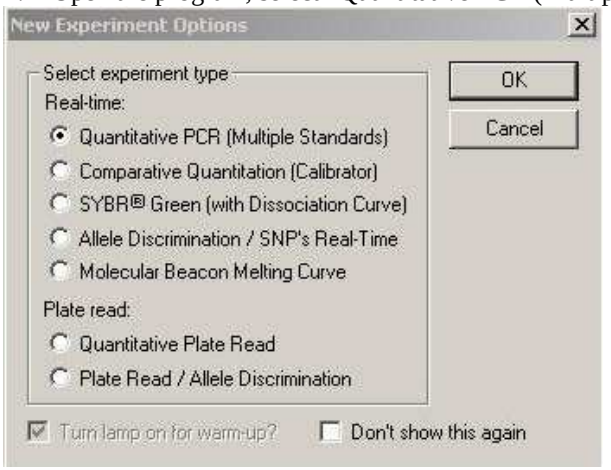
Results analysis for **HEX** channel (HCV RNA)

Activate the button "**Log View**". Put the threshold line (with the left button of the mouse) at such level where the curves of fluorescence are linear (see figure)

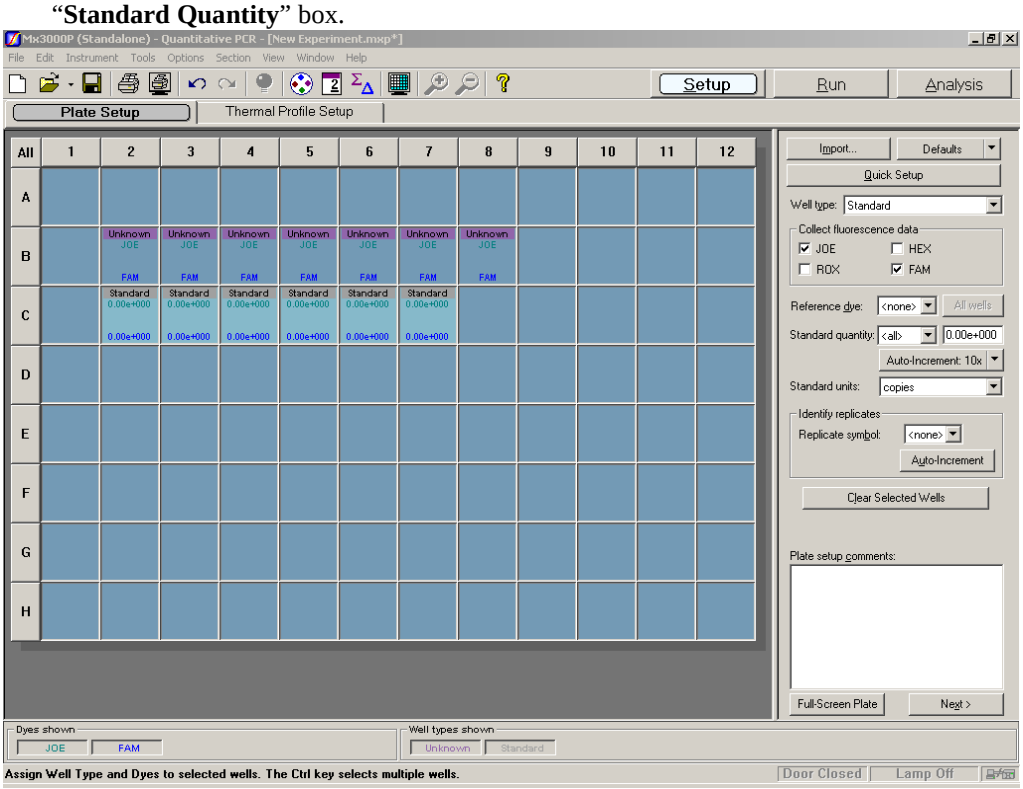


MX3000P® and MX3005P® (Stratagene)

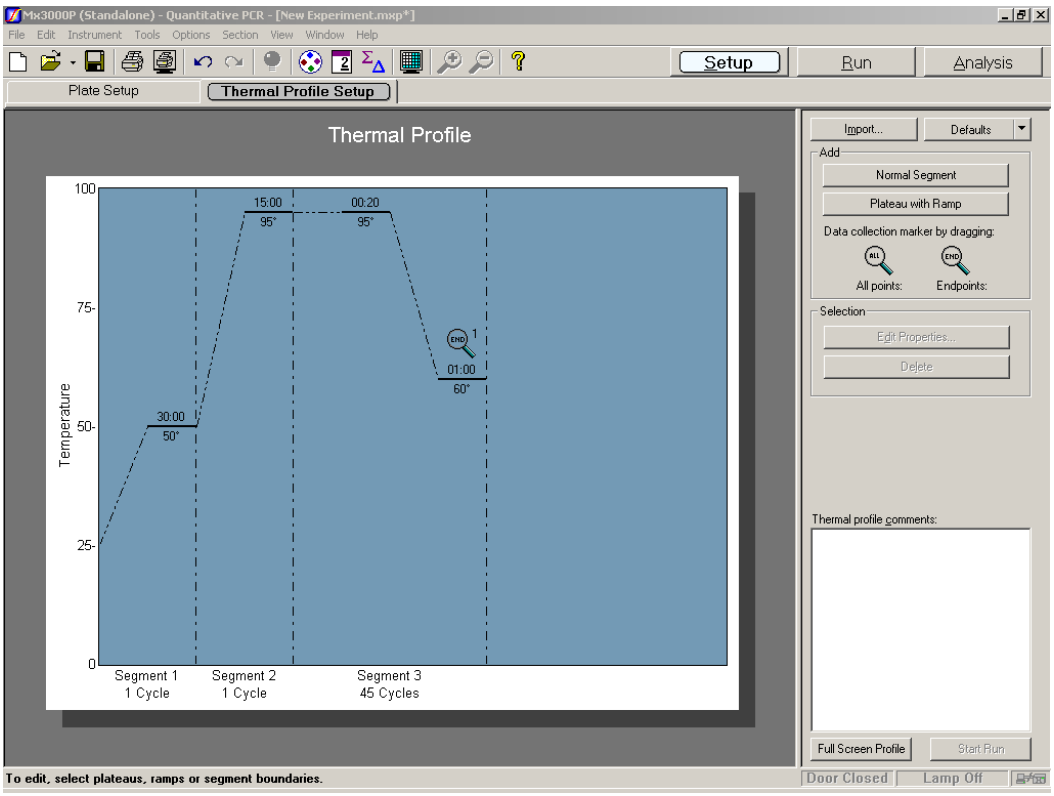
1. Open the program, select “Quantitative PCR (Multiple Standarts)” and click “OK”



2. At the top left of the window choose “Plate Setup”
3. In the window “Well type” set “Unknown” for the samples and “Standard” to identify calibrators.
4. In the window “Collect fluorescence data” select for all samples the channels Fam and Joe.
5. Enter the concentrations of the Quantitative Standards (reported on the HCV TM MX Quant Data Card) in the “**Standard Quantity**” box.

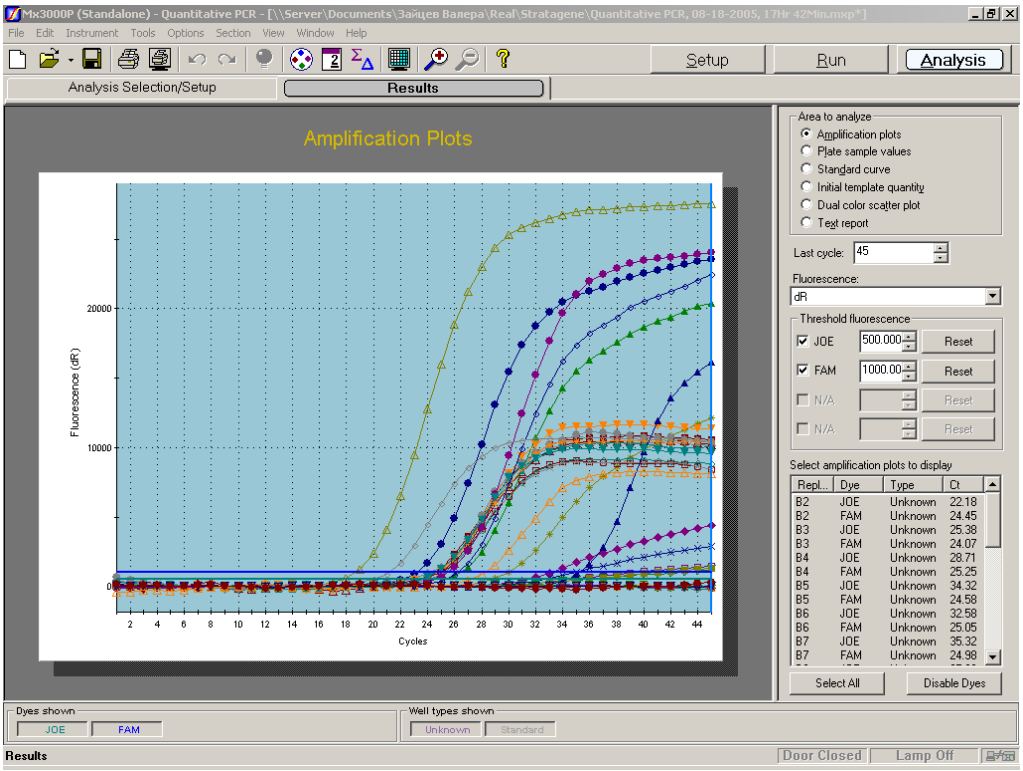


6. At the top left of the window select button “Thermal Profile Setup”
7. Set the following parameters of amplification:
    1. 50°C – 30 min
    2. 95°C – 15 min
    3. 95°C – 20 sec
       60°C – 60 sec
       45 Cycles
8. Fluorescence is measured at 60°C. To do this, set on the Thermal Profile graph the “Endpoints” marker.
9. Click “Run” button, enter a name for the experiment and save it.



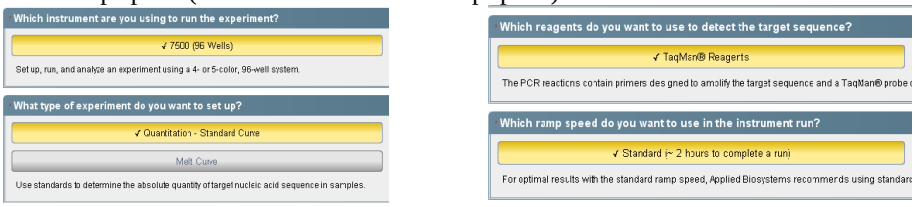
Data Analysis

1. Soon after the amplification is over, choose the button “Analysis” at the top left of the window .
2. Choose the button “Results”
3. At the right angle of the window “Area to analyze” select “Amplification plots”.
4. Set “Threshold fluorescence” **500** for the Joe channel and **1000** for the Fam channel.
5. In the window “Text report” appear for each sample the values of Ct and experimental values of copies cDNA HCV and cDNA IC.
6. Take care that the value of RSq (correlation coefficient) in the window “Standard curve” is not lower than 0,9 for both channels.

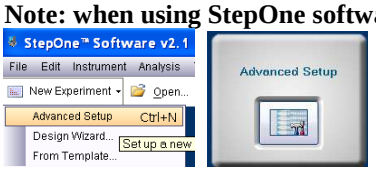


Applied Biosystems® 7300/7500/StepOne Real Time PCR Systems (Applera)

1. Select in the main menu the option "**New Experiment**" (*Advanced Setup*), insert experiment details. Select your Instrument Type (*7500 / 7500 fast/StepOne/StepOne Plus*), *Quantitation – Standard curve*, *Taqman Reagents*, *Standard* ramp speed (DO NOT select Fast RampSpeed).

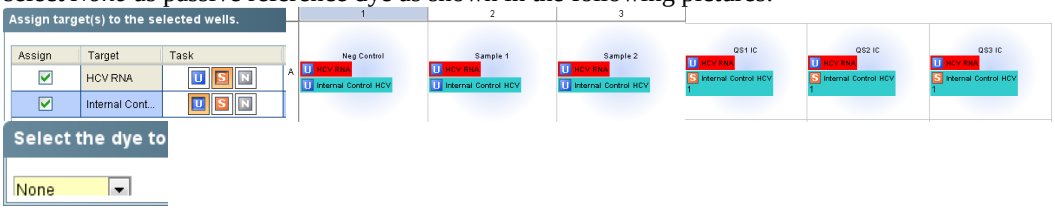

Which instrument are you using to run the experiment?
✓ 7500 (96 Wells)
Set up, run, and analyze an experiment using a 4- or 5-color, 96-well system.
What type of experiment do you want to set up?
✓ Quantitation - Standard Curve
Melt Curve
Use standards to determine the absolute quantity of target nucleic acid sequence in samples.
Which reagents do you want to use to detect the target sequence?
✓ TaqMan® Reagents
Which ramp speed do you want to use in the instrument run?
✓ Standard (~ 2 hours to complete a run)

Note: when using StepOne software, be sure to click New Experiment – Advanced Setup

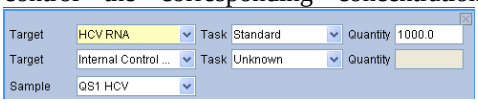


2. Click

, add two targets: one for *HCV* (Reporter ***Joe***) and the other one for Internal Control (Reporter ***Fam***); select Quencher – ***None*** for both targets.
3. Click *Add new samples* multiple times and enter name for all samples, controls and standards
4. Click **Assign target and Samples** tab, associate samples ( ) and standards ( )with the two targets and select *None* as passive reference dye as shown in the following pictures:



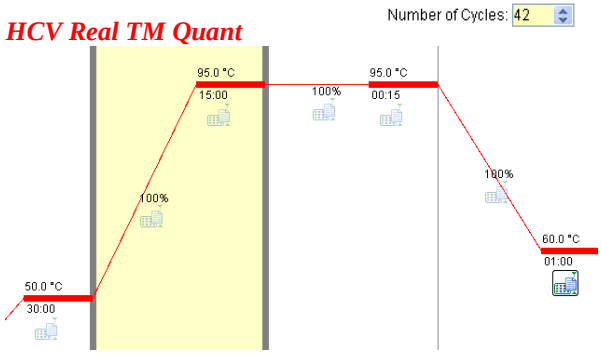
5. Double-click on each standard well (a new small window will appear) and enter both for HCV and Internal control the corresponding concentration in the *Quantity* field as indicated in the Data Card:



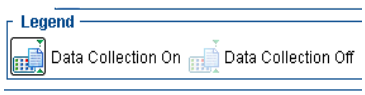
6. Click on

, insert Reaction volume (25 µl):

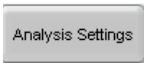
HCV Real TM Quant



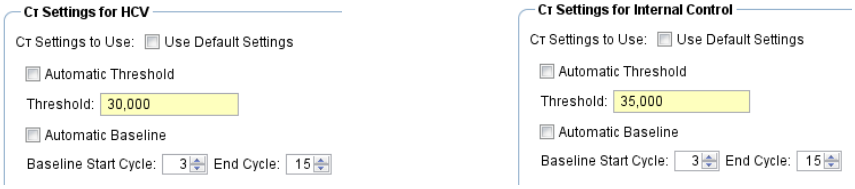
Stage	Profile	Reps
1	50°C – 30:00	1
2	95°C – 15:00	1
3	95°C – 0:15 60°C – 1:00 *	42



7. Click

to begin PCR reaction.
8. After the run is complete, select all samples and standards and click 

9. Deselect *Use default settings*, *Automatic threshold* and *automatic baseline*; suggested values are*:



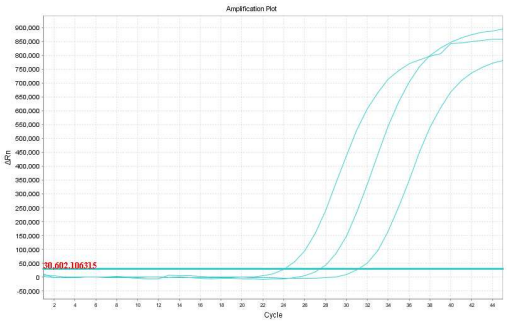
** for StepOne instrument recommended Threshold values are: Joe (HCV) = 1,000, Fam (IC) = 2,000.*
NOTE: please slightly adapt suggested values according to your result curves

10. Click

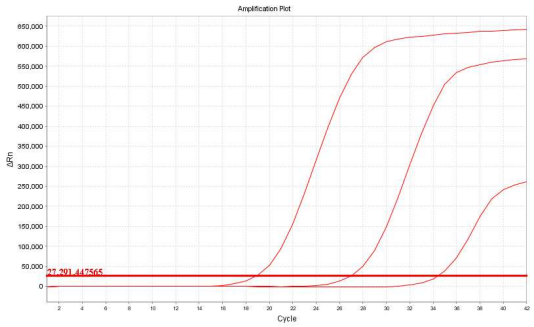
11. Select all samples and standards, click *View well table* and then Click *File -> Export*, select *Results* only.

12. Click Customize Export and sort samples by reporter, as indicated in the above image. Then click

to export results in a file.
13. Using the exported file, calculate results pasting data from the *Sample* and the *Quantity* columns in the provided *HCV Quant Result Calculation* sheet.



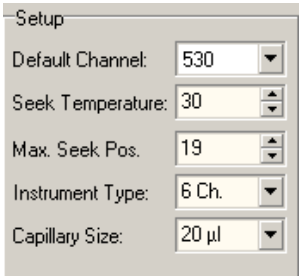
FAM – Internal Control



Joe – HCV

LightCycler 2.0® (Roche)

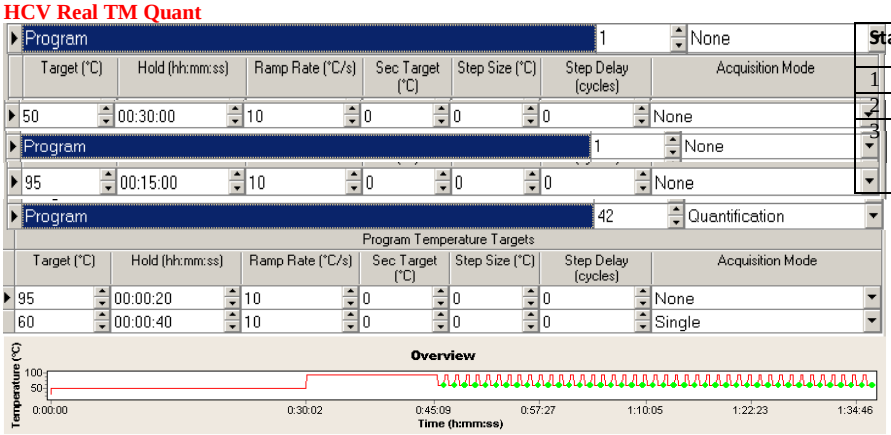
1. Select "**New", "LightCycler Experiment**", Click and insert as follows:



- Max. Seek Pos. indicates the number of samples used in the experiment
- Choose Instrument Type according to your instrument
- Choose capillary Size according to your capillaries (we suggest to select 20 µl and use 30 µl polycarbonate capillaries)

2. Program the thermalcycling as follows:

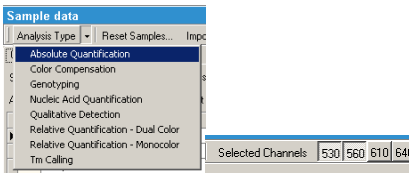
HCV Real TM Quant


Program 1 None
Target (°C) Hold (hh:mm:ss) Ramp Rate (°C/s) Sec Target (°C) Step Size (°C) Step Delay (cycles) Acquisition Mode
50 00:30:00 10 0 0 0 None
Program 1 None
95 00:15:00 10 0 0 0 None
Program 42 Quantification
Program Temperature Targets
Target (°C) Hold (hh:mm:ss) Ramp Rate (°C/s) Sec Target (°C) Step Size (°C) Step Delay (cycles) Acquisition Mode
95 00:00:20 10 0 0 0 None
60 00:00:40 10 0 0 0 Single
Overview
Temperature (°C)
100
50
0:00:00
0:30:02
0:45:09
0:57:27
1:10:05
1:22:23
1:34:46
Time (h:mm:ss)

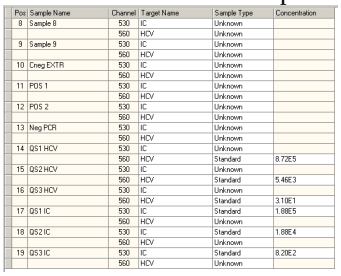
Stage	Profile	Reps
1	50°C – 30:00	1
2	95°C – 15:00	1
3	95°C – 0:15 60°C – 0:40 *	42

*Acquisition mode - *Single*

3. Click and insert sample names; then select *Absolute Quantification* from *Analysis Type* and select *530, 560* in Selected Channels section:

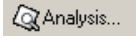


4. Insert target Name, Sample Type (*Standard* for standards, *Unknown* for all other samples), and insert sample concentration values as reported in the Data Card:



Pos	Sample Name	Channel	Target Name	Sample Type	Concentration
8	Sample 8	530	IC	Unknown	
		560	HCV	Unknown	
9	Sample 9	530	IC	Unknown	
		560	HCV	Unknown	
10	Cneg EXTR	530	IC	Unknown	
		560	HCV	Unknown	
11	POS 1	530	IC	Unknown	
		560	HCV	Unknown	
12	POS 2	530	IC	Unknown	
		560	HCV	Unknown	
13	Neg PCR	530	IC	Unknown	
		560	HCV	Unknown	
14	QS1 HCV	530	IC	Unknown	
		560	HCV	Standard	8.72E5
15	QS2 HCV	530	IC	Unknown	
		560	HCV	Standard	5.46E3
16	QS3 HCV	530	IC	Unknown	
		560	HCV	Standard	3.10E1
17	QS1 IC	530	IC	Standard	1.88E5
		560	HCV	Unknown	
18	QS2 IC	530	IC	Standard	1.88E4
		560	HCV	Unknown	
19	QS3 IC	530	IC	Standard	8.20E2
		560	HCV	Unknown	

5. Click to start PCR reaction.

6. After Run is completed click  button and select Absolute quantification. Select Method (Auto) , Standard Curve (In Run). Ct values and calculated results will appear, for the selected channel, under the *CP* and *Concentration* column in the Absolute Quantification table.

Absolute Quantification [HCV-Quant-FAM-JOE

Channel (560) ▾ Color Compensation (On) ▾ Program

Samples			Results		
I...	C...	P...	Name	CP	Concentration	Standard
☑		1	Sample 1			
☑		2	Sample 2	32.92	[2.40E1]	
☑		3	Sample 3	36.69	[1.29E0]	
☑		4	Sample 4	31.98	4.96E1	
☑		5	Sample 5	31.17	9.27E1	
☑		6	Sample 6	31.72	6.06E1	
☑		7	Sample 7	31.59	6.67E1	
☑		8	Sample 8	31.84	5.53E1	

7. Calculate results manually using the usual formula as indicated in the User Manual of the kit.

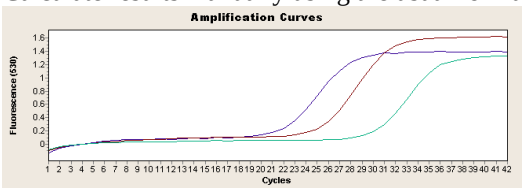


Fig. 1 FAM / 530 channel (Internal Control)

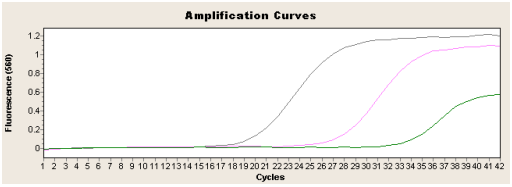


Fig. 2 Joe / 560 channel (HCV)

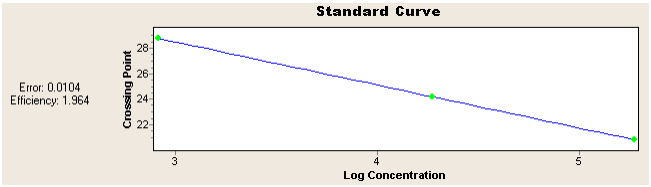
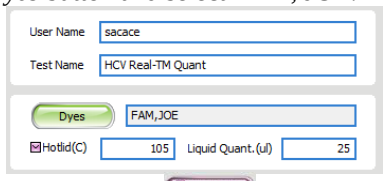


Fig. 3 Standard Curve

At first usage the operator must perform a color compensation experiment asking Sacace for the detailed instructions.

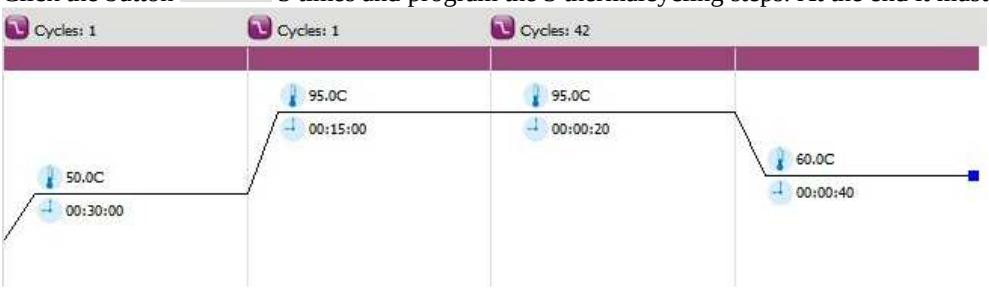
LineGene K® (Bioer)

Open LineGeneK Software. Click *File -> New*. In the *Setup Programs* tab, insert *User Name* and *Test Name*. Click *Dyes* button and select FAM, JOE. Insert the correct liquid quantity (25 µl).

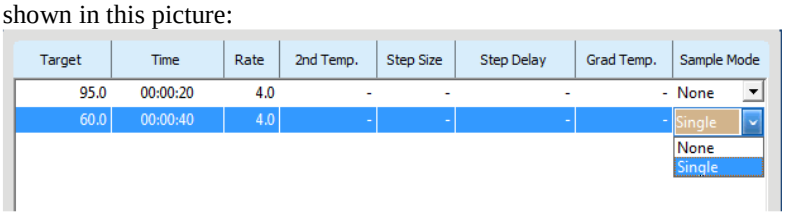


Click the button

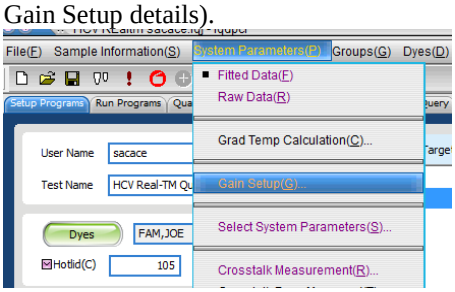
3 times and program the 3 thermalcycling steps. At the end it must look like this:

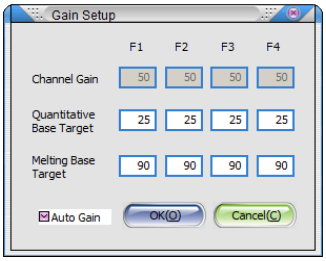


On the second phase (60.0 °C) of step 3 select "*Single*" on the *Sample Mode* column to collect fluorescence data, as shown in this picture:

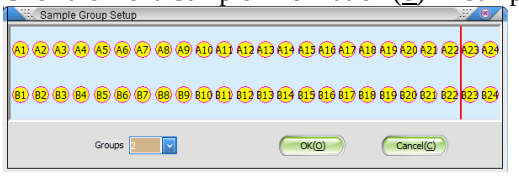


Click the menu System Parameters (P) -> Gain Setup (G). Select Auto Gain box and click OK (see picture below for Gain Setup details).

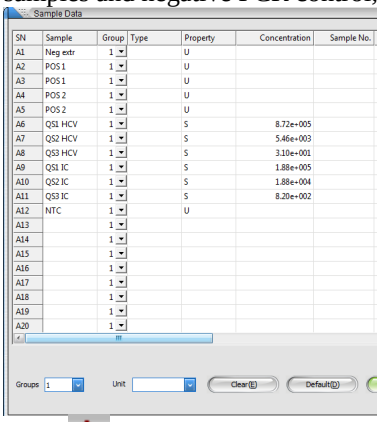




Click the menu Sample Information (S) -> Sample Group Setup…(R). Select at least 2 groups.



Click the menu Sample Information (S) -> Sample Data…(S). Insert Sample name, Property (U for unknown samples and negative PCR control, S for Standards) and insert standard concentration reported in Data Card.



Click or click File -> Run Programs (R) to Run the PCR program.

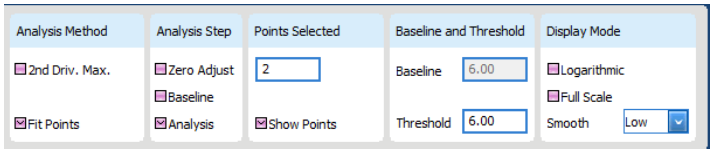
Results Analysis - FAM

In the *Quantitative Analysis* tab, select from the menu Dyes(D) -> FAM(F). Select standard Concentration boxes (left mouse click+CTRL) for Internal Control (see picture below).

SN	Color	Group	Sample	Concentration
A1		1	Neg extr	
A2		1	POS 1	
A3		1	POS 1	
A4		1	POS 2	
A5		1	POS 2	
A6		1	QS1 HCV	8.72e+005
A7		1	QS2 HCV	5.46e+003
A8		1	QS3 HCV	3.10e+001
A9		1	QS1 IC	1.88e+005
A10		1	QS2 IC	1.88e+004
A11		1	QS3 IC	8.20e+002
A12		1	NTC	

Then do in this order:

1) Under Analysis Method select *Fit Points*.
2) Under Analysis Step Click *Zero Adjust*, select *Auto* and then click *OK*. Then click *Baseline*
3) Select 2 points and set the base line (drag and drop the red baseline) as low as possible but above the noise of each sample (usually 6-8)
4) Under Analysis Step click *Analysis*. Calculated copies/reaction of Internal Control and Ct Results should appear in the *Ct* and the "*Calculated Concentration*" column respectively.



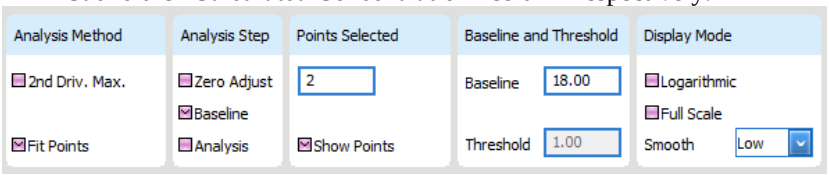
Results Analysis - Joe

In the *Quantitative Analysis* tab, select from the menu Dyes(D) -> Joe(F). Select standard Concentration boxes (left mouse click+CTRL) for HCV.

SN	Status	C.	Sample	Concentration
A1			Neg extr	
A2			POS 1	
A3			POS 1	
A4			POS 2	
A5			POS 2	
A6			QS1 HCV	8.72e+005
A7			QS2 HCV	5.46e+003
A8			QS3 HCV	3.10e+001
A9			QS1 IC	1.88e+005
A10			QS2 IC	1.88e+004
A11			QS3 IC	8.20e+002
A12			NTC	

Then do in this order:

1) Under Analysis Method select *Fit Points*.
2) Under Analysis Step Click *Zero Adjust*, select *Auto* and then click *OK*. Then click *Baseline*
3) Select 2 points and set the base line (drag and drop the red baseline) as low as possible but above the noise of each sample (usually 15-20)
4) Under Analysis Step clic Analysis. Calculated copies/reaction of HCV and Ct Results should appear in the *Ct* and the "*Calculated Concentration*" column respectively.



Eco Real Time PCR System (Illumina®)

1. Open Eco software, click

and under *Experiment Type* select

,

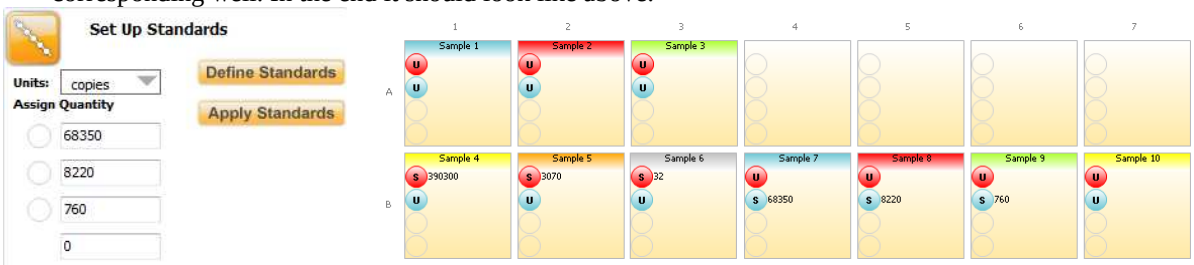
, select RNA as starting material for HCV Real-TM Quant.

2. Under Quantification Method select

, and insert experiment name. Click

3. Click , set up two assays: one for *HCV* (Reporter ***HEX***) and the other one for Internal Control (Reporter ***Fam***); select Quencher – ***Non fluorescent*** for both targets. Click

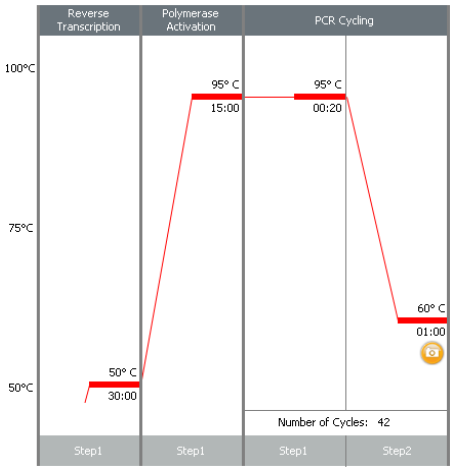
4. Click and enter name for all samples, controls and standards. Click
5. Associate samples and standards with the two previously designed assays following this procedure: select the well, select *Unknown* for samples and *Standard* for standards and to assign the corresponding assay click the white circle of the HCV and Internal control assays (the circle will become colored): for each well both Assays must be assigned.
6. Click and in the Set Up Standards box insert each value reported in the DataCard (click Enter after inserting each value) both for HCV assay and Internal control assay; then associate concentration values to the corresponding well. In the end it should look like above:



7. Click on

, and program the instrument as follows (reaction volume is 20 µl**):

HCV Real TM Quant



Stage	Profile	Reps
1	50°C – 30:00	1
2	95°C – 15:00	1
3	95°C – 0:15 60°C – 1:00 *	42

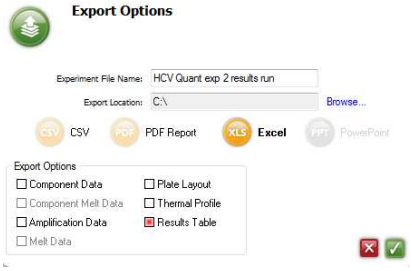
** Add **10 µl** of **Reaction Mix** into each tube.
Add **10 µl** of **extracted RNA** sample or quantitative standards to the appropriate tube with Reaction Mix

8. Click *Start Run* to begin PCR reaction.
9. After the run is complete click

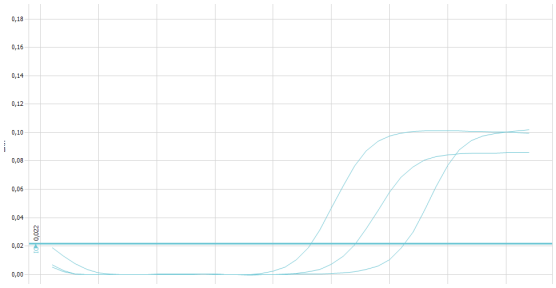
then click

tab to interpret the results.

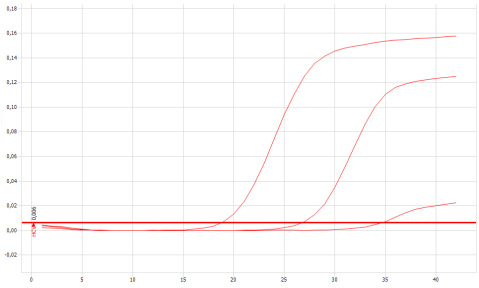
10. Click to Export the samples, in the *Export Options* select *Results Table*



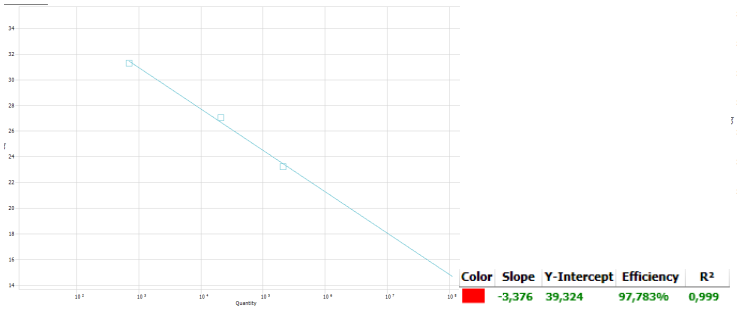
11. Using the exported file, calculate results pasting data from the *Sample Name* and the *Quantity* columns in the provided *HCV Quant Result Calculation* sheet.



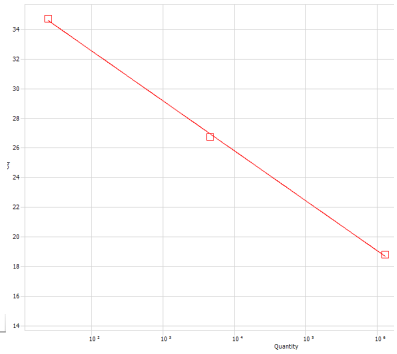
FAM – Internal Control



Joe – HCV, HBV



Standard Curve



Standard Curve

15. RESULTS INTERPRETATION

The Internal Control (IC) is detected on the FAM channel and HCV RNA on the Joe/HEX/Cy3 channel.

For each control and patient specimen, calculate the concentration of HCV RNA using the following formula:

$$\frac{\textbf{HCV DNA copies/specimen (Joe/HEX/Cy3 channel)}}{\textbf{IC DNA copies/specimen (FAM channel)}} \textbf{ x coefficient* = IU HCV/mL}$$

*coefficient is specific for each lot and reported in the HCV TM RG Quant Data Card provided in the kit. Results may by calculated also using "HCV Quant Result Calculation sheet" provided with the kit

To obtain the results in copies/mL multiply the IU HCV/ml value by 4:

IU RNA HCV/mL x 4 = copies RNA HCV/mL

16. PERFORMANCE CHARACTERISTICS

The sensitivity, the specificity, the performance and the linearity of the kit HCV Real-TM Quant were determined with the following samples:

1. The HCV RNA standard with the concentration of $2x10^5$ IU/ml and its dilution.
2. The samples of blood plasma obtained from 215 patients with IgG antibodies to HCV. The results were compared to those ones obtained by the reference manufacturer's kit.
3. 80 samples of blood plasma obtained from donors..

Analytical specificity

The analytical specificity of the primers and the probes was validated with 80 negative samples. They did not generate any signal with the specific HCV primers and probes. The specificity of the kit **HCV Real-TM Quant** was 100%
The potential cross-reactivity of the kit **HCV Real-TM Quant** was tested also against the group control listed in the following table. It was not observed any cross-reactivity with these pathogens.

Table 1: Testing the specificity of the kit with other pathogens:

Control group	Results
Adenovirus type 2	-
Adenovirus type 3	-
Adenovirus type 7	-
Cytomegalovirus	-
Epstein Barr virus	-
Hepatitis B virus	-
Human immunodeficiency virus 1	-
Herpes simplex virus 1	-
Herpes simplex virus 2	-
Human herpes virus 6	-
Human herpes virus 8	-
HPV groups β, γ, μ (1,3,4,5,8,37,38,65,20,24,49,50,15)	-
HPV group α (6,11,26,53,7,27,10)	-

Analytical sensitivity

The kit **HCV Real-TM Quant** allows to detect HCV RNA in 100% of the tests with a sensitivity not less than 20 IU/ml (value obtained using the "Magno-Virus" extraction kit Sacace REF K-2-16 and RotorGene 6000). The detection was carried out on the control standard and its dilutions by negative plasma.

Linearity

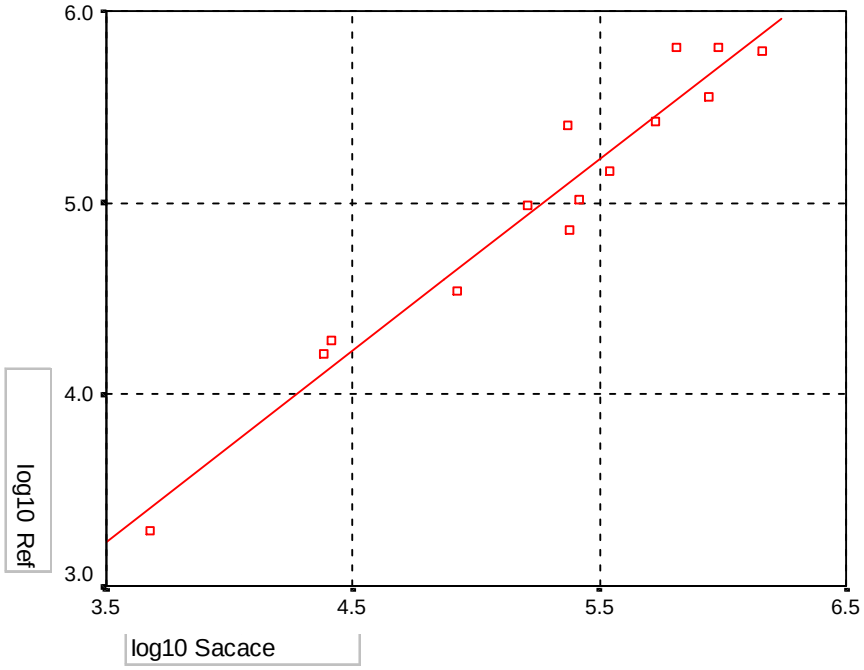
The linearity of the HCV Real-TM Quant assay was tested with the HCV RNA Standard and it's dilution using HCV-negative human plasma. Each dilution was analysed three times and the mean HCV RNA titer of each sample was determined.
HCV Real-TM Quant is linear from 2,5 x 10^2 to 5 x 10^7 IU/mL. Test results greater than 50.000.000 IU/mL are above the upper limit of quantitation of the test and should be reported as "greater than 50.000.000 IU/mL". If quantitation results are desired for such samples, the specimen should be diluted 1:10 with negative serum and retested. Test results less than 250 IU/mL are below the lower limit of quantitation of the test and should be reported as "less than 250 IU/mL".

Table 1. Comparison among the results of HCV RNA viral load detection using "HCV Real-TM Quant" (Sacace) kit and reference manufacturer's kit.

Sample	Reference		HCV Real-TM Quant		Δ Log_{10} Reference Log_{10} Sacace
	IU/ml	Log_{10}	IU/ml	Log_{10}	
N026	640.000	5.81	955.000	5.98	**-0.17**
B092	354.000	5.55	874.000	5.94	**-0.39**
C009	19.200	4.28	26.000	4.41	**-0.13**
P110	621.000	5.79	1460.000	6.16	**-0.37**
N075	16.400	4.21	23.800	4.38	**-0.17**
H069	97.100	4.99	164.000	5.21	**-0.22**
A152	147.000	5.17	343.000	5.54	**-0.37**
D018	250.000	5.40	235.000	5.37	**0.03**
D108	643.000	5.81	644.000	5.81	**0.00**
D159	266.000	5.42	540.000	5.73	**-0.31**
H037	105.000	5.02	262.000	5.42	**-0.40**
S033	72.100	4.86	209.000	5.32	**-0.46**
B038	34900	4.54	82800	4.92	**-0.38**
E100	1960	3.29	4.810	3.68	**-0.39**
D036	>850.000	>5.93	19.700.000	7.29	-
Standard	250.000	5.39	270.000	5.43	**-0.04**
Standard1	120.000	5.07	135.000	5.08	**-0.01**
Standard 2	11.000	4.04	12.000	4.04	**0.00**
Standard 3	1.000	3.00	1.120	3.04	**-0.04**
Standard 4	<500	<2.7	<500	<2.7	-

In the above table the data show that almost all the results obtained from the clinical and control samples with HCV Real-TM Quant test differ by less than 0.5 Log_{10} from the values obtained with Reference manufacturer's kit. The figure 2 illustrates the graphic correlation between the HCV RNA viral load measured by two tests. The correlation factor is 0,975 ($p_v < 0,01$).

Figure 2. Graphic comparison among the results obtained with “HCV Real-TM Quant” (Sacace) kit and reference manufacturer’s kit.



17. TROUBLESHOOTING

1. Weak (Ct > 34) signal of the IC (Fam channel): retesting of the sample is required.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended RNA extraction method and follow the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting the extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and for the IC detection select the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the RNA extraction procedure.
2. Weak (Ct > 40) signal on the Cy3 channel: retesting of the sample is required.
3. The correlation coefficient R^2 is less than 0.9: retesting of all samples is required.
4. The calculated concentrations of HCV Rec Pos1 and/or HCV Rec Pos2 are different from given control concentrations, reported in the Data Sheet: retesting of all samples is required.
5. Any signal on the Joe/HEX/Cy3 channel with Negative Control of extraction.
    - Contamination during RNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips among tubes.
        - ⇒ Repeat the RNA extraction with the new set of reagents.
6. Any signal with Negative PCR Control.
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive controls at the end.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

18. REFERENCES

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19. EXPLANATION OF SYMBOLS

REF Catalogue Number

RUO For *Research Use Only*

LOT Lot Number

Expiration Date

Contains reagents

Caution!

VER Version

Manufacturer

Temperature limitation

* Cycler™ and iQ5™ are trademarks of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Corbett Research
*MX3000P® and MX3005P® are trademarks of Stratagene
*Applied Biosystems® is trademarks of Applera Corporation
* SmartCycler® is a registered trademark of Cepheid
* LightCycler® is a registered trademark of Roche
* LineGene K® is a registered trademark of Bioer
* Eco PCR Real Time System® is a registered trademark of Illumina

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*PCR: The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffmann-La Roche and applicable in certain countries. Sacace does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this kit is recommended for persons that either have a license to perform PCR or are not required to obtain a license